RECOMBINANT HUMAN BUTYRYLCHOLINESTERASE BIOPROCESS DEVELOPMENT IN CHINESE HAMSTER OVARY CELLS

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ABSTRACT

The predominant form of human butyrylcholinesterse (BChE) in blood is a tetramer complex of four identical subunits. While, the recombinant BChE in Chinese hamster ovary (CHO) cells consists mainly dimers and a small amount of monomers. In current biopharmaceutical industry, mammalian cell culture platforms, especially CHO cells, are predominantly used for the production of therapeutic glycoproteins.

We have developed an efficient BChE expression system in CHO cells by exploring various combinations of different promoters, codon optimization effect and 6xhistidine-tag effect. Then we transfected three proline-rich sequences of different proline lengths (P8, P14 and P24) respectively into recombinant human BChE (rhBChE) stably expressing cell line and determined individual's isomer distribution by native electrophoresis and enzymatic activity by Ellman assay. P24 was able to organize more than 60% of rhBChE-his into tetramers. We tested BChE purification efficiency by using Ni-NTA purification system. To further enhance the tetramer content in rhBChE expressing cell line, we used fluorescence-activated cell sorting method, which has remarkably increased P24 sequence expression level. In this thesis, we have performed a bioprocess to produce recombinant huBChE tetramers in Chinese Hamster Ovary (CHO) Cells. We have created two cell lines from CHO that will be further studied and optimized for the production of rhBChE: CHO-rhBChE, CHO-rhBChE-P24.

Advisor: Dr. Michael Betenbaugh

Reader: Dr. Kevin Yarema

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 HUMAN BUTYRYLCHOLINESTERASE STRUCTURE AND FUNCTION

Butyrylcholinesterase (BChE) is a serine hydrolase which has gained a lot attention because it hydrolyzes cocaine, heroin, aspirin, bambuterol and succinylcholine, and it scavenges organophosphorus pesticides and chemical warfare agents, which is also called organophosphorus bioscavenger[1-6].

Amino acid sequencing of the mature BChE enzyme isolated from human plasma revealed that each subunit comprise 574 residues with a molecular mass of 85 000 Da and nine N-linked carbohydrates [7]. Reports pointed out that pharmacokinetic properties of the recombinant BCHE are affected by both glycosylation and size of the recombinant protein [8, 9]. Oligosaccharide profiles of recombinant human BCHEs showed significant difference compared with native plasma enzyme [8, 10]. But the most important characteristic for the bioscavenger, i.e., half-life [11], is mainly dependent on the size of the protein [12].

The predominant form of human BChE in blood is a tetrameric complex of four identical subunits, as shown in Figure 1. To date, BChE comes as a single variant: BChET, which possesses a C-terminal t-peptide similar to that of AChET (acetylcholinesterase) [13, 14]. The t-peptide of BChET is called the tryptophan amphiphilic tetramerization (WAT) domain, containing seven strictly conserved aromatic residues (Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564), including three evenly spaced tryptophans and is organized as a α-helix [15]. WAT domain is located within the last 40 C-terminal residues [16]. These seven residues are responsible

for stabilizing BChE and mediating the interactions that increase tetrameric assembly when BChE subunits are interacting with its tetrameric partners.

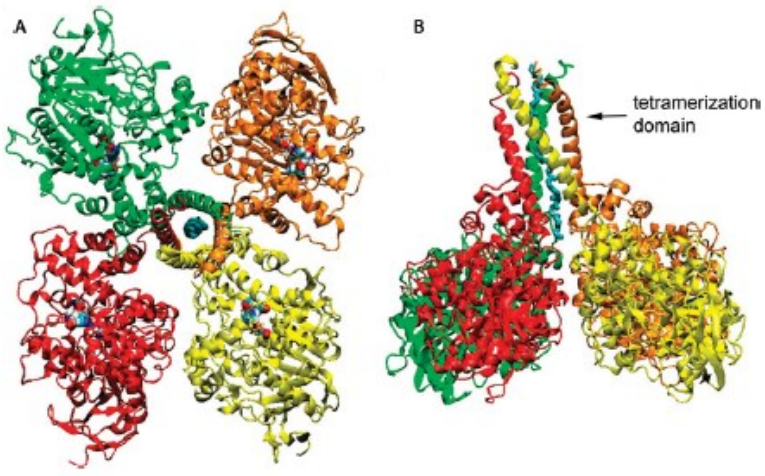


Figure 1. Structural model of the human BChE-tetramers showing with the polyproline peptide located in the core of the tetramerization domain. (A) Human BChE viewed from the top. (B) Human BChE tetramer viewed from the side [17].

The half-life of BChE (wild type or mutant) in circulation is dependent on the quaternary form of the protein. The residence time of the monomer is only on the order of minutes in the circulation of mice, whereas native human BChE has a half-life of ~46 h in mice [8, 18, 19] or ~12 days in human plasma [20]. It has been shown that the predominant forms of recombinant BChE secreted from Chinese hamster ovary (CHO) cells were monomers and dimers, whereas native BChE consists more than 95% of tetramers that lead to the longer half-life of native BChE in plasma [14, 17, 19].

According to the studies about BCHE, the tetrameric forms of BChE are inserted in the basal lamina of neuromuscular junctions or anchored in cell membranes through the interaction of four C-terminal peptides with proline-rich attachment domains (PRADs) of cholinesterase-associated collagen Q or of the transmembrane protein PRiMA (proline-rich membrane anchor) [21, 22], as depicted in Figure 2. ColQ and

PRiMA differ in the length of their proline-rich motifs (10 and 15 redidues, repectively) (Fig2). Duysen et al. [19] reported that coexpression in CHO cells of BChE and PRAD of ColQ yielded 70% tetrameric BChE and 30% monomer and almost no dimer. While Ilyushin et al. [12] reported coexpression of rhBChE and PRAD of ColQ in CHO cells showed BChE 50% dimers and 50% tetramers primarily expressed and no monomer detectable in the culture medium. Furthermore, He li et al [21]extracted a series of proline-rich peptides from denatured human and horse plasma BChE, which have the same proline-rich core sequence PSPPLPPPPPPPPPPPPPPPPLP (mass 2663 Da) but varied in length at their N- and C-termini. Collectively, hundreds of reports have demonstrated that the proline-rich peptides organize the 4 subunits of BChE into a 340 kDa tetramer by interacting with the C-terminal tetramerization domain of each subunit.

PRAD of ColQ (P8): CCLLTPPPPPLFPPPFF
PRAD of PRiMA(P14): CQCRPPPPLPPPPPPPPPRLL
The core proline-rich sequence extracted from human plasma (P24):
PSPPLPPPPPPPPPPPPPPPPPLP

Figure 2 Three proline-rich sequences, written with the N-terminus on the left and the C-terminus on the right. PRAD of ColQ is abbreviated as P8, which has 8 proline residues; PRAD of PRiMA is abbreviated as P14, which has 14 proline residues; the core proline-rich sequence extracted from human plasma is abbreviated as P24, which has 24 proline residues.

Moreover, Marilynn A. Larson et al. [23] reported that 90-98% of purified rBChE (65 uM) could be assembled into tetramers when incubated with synthetic 17-mer or 50-mer polyproline peptides (100uM) for 1.5h at 25℃. Thus, the process of assembling active BChE into tetramers could occur in vitro in the presence of appropriate BChE and polyproline peptide concentrations.

However, the published results of coexpression of rBChE and PRAD of ColQ is self-controversial and it has been reported that rBChE tetramerization was inefficient with smaller 8-mer polyproline peptide [23]. Additionally, no documents reported the performance of introduction of PRAD of PRiMA and the proline-rich core sequence extracted from human BChE into CHO BChE expressing cell line *in vivo*. Plus, *in vivo* assembly could avoid the high concentrations of rBChE and polyproline peptide required for in vitro assembly.

Therefore, developing a CHO expression system to force *in vivo* tetramerization of recombinant BChE would be beneficial. In this study, I first developed an efficient BChE expression system in CHO cells, and then introduced three Polyproline-rich sequences (P8: PRAD of ColQ, P14: PRAD of PRiMA and P24: proline-rich core sequence extracted from human BChE) respectively into the CHO-K1 cells expressing recombinant human BChE.

1.2 Ni-NTA purification of rhBChE

A common used approach to purify rhBChE is to use BChE affinity on procainamide, which is a small molecule that binds strongly on the enzyme's substrate binding site [24]. However, some BChE variants, like G117H/E197Q BChE, have little affinity for procainamide, and thus are not efficient purification using a procainamide column [25, 26]. Therefore, there is a need for a robust BChE purification method regardless of the rhBChE's three-dimensional structure. Affinity tags are a logical choice [25].

A polyhistidine-tag is an amino acid motif consisting of at least six histidine (His) residues, often added at the N- or C-terminus of the target protein. Histidine exhibits a

very strong interaction with metal ions like Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}. For instance, his-tagged protein can bind to the Ni-NTA groups on the matrix with an affinity for greater than that of anti-body antigen or enzyme-substrate interactions. And a widely employed protein purification method utilizes immobilized metal-affinity chromatography (IMAC) to purify recombinant protiens containing a polyhistidine-tag [27].

1.3 Fluorescence activated cell sorting

Fluorescence-activated cell sorting (FACS) is a specialized application of flow cytometry. It provides a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time, based upon the specific fluorescent characteristics of each cell. It is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest [28].

The use of internal ribosome entry sites (IRESs) in dicistronic expression vectors enables the expression of two genes controlled by one promoter in target cells [29]. Usually, IRES is linked between a target gene and an autofluorescent reporter gene such as green fluorescent protein (GFP) to create a dicistronic vector. Thus, the construct of P24 linked with IRES-GFP would be valuable. And the use of FACS provides a higher efficiency to select P24 highly expressing cells compared to antibiotic selection method.

CHAPTER 2: MATERIALS AND METHODS

2.1 CONSTRUCTION OF EXPRESSION VECTORS

Gene constructs, pcDNA3.1/v5-his-TOPO-huBChE, was kindly obtained from Dr. Ashima Saxema at the Walter Reed Army Institute of Research in Silver Spring, MD. The DNA fragment encoding the open reading frame of huBChE (human BChE) was amplified by PCR using a 5' primer containing an *EcoRV* site, 5'-GCCGATATCGCCACCATGGATAGCAAAGTCACAATCA-3' and a 3' primer containing a PacI site, 5'-GCCTTAATTAACTATTATCAAGACCCACACAACTTTCTTTC-3', and the pcDNA3.1/v5-his-TOPO-huBCHE construct as a template. The sequences of human BChE and P8, P14 and P24 were codon optimized to fit expression in CHO cells from Biomatik. BChE-his was constructed by fusing the C-terminus of BChE with a 6x his tag. A set of BChE combinations including BChE, BChE-his, Codon-optimized BChE(O-BChE) and Codon-optimized BChE-his (O-BChE-his), were inserted into pcDNA3.1 vector though HindIII and BamHI separately. The same set of BChE combinations (BChE, BChE-his, O-BChE, O-BChE-his) were inserted into pEF6/V5-his TOPO TA vector (Addgene) through BamHI and NotI and pCHO1.0 vector (Life Technologies) through EcoRV and PacI respectively. These vector constructs are shown in Figure 3.

Besides, pcDNA-PRAD plasmids were constructed by inserting the corresponding proline-rich sequences (P8, P14 and P24) fused with a flag tag into pcDNA3/Hgy(+) through HindIII and BamHI sites individually.

For pcDNA3.1 P24-IRES-GFP construct, codon optimized P24 sequence was introduced into pcDNA3.1 vector through NheII and HindIII sites followed by IRES

sequence inserted between HindIII and NotI sites. Then, GFP sequence was inserted between NotI and Xhol sites right after IRES sequence.

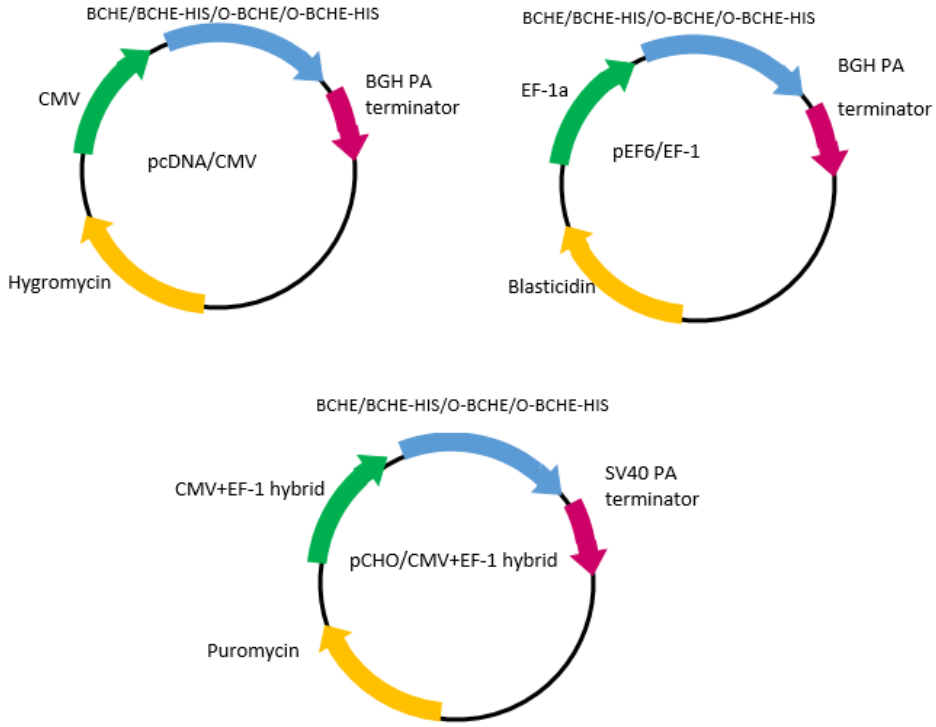


Figure 3 Expression vectors used in developing an efficient rhBChE-his system.

2.2 Cell Culture and Transfection

Adherent CHO-K1 cell lines (Sigma-Aldrich, St. Louis, MO) were maintained in adherent growth flasks (Sarstedt, Numbrect, Germany) in Ham's F-12K (Life Technologies, Carlsbad, CA) Medium supplemented with 10% fetal bovine serum, FBS (Thermo Scientific, Logan, UT), 20 mM L-glutamine (Corning Cellgro Manassas, VA), and 1% non-essential amino acids (Invitrogen, Carlsbad, CA). Cells were grown at 37oC with 5% CO2 in a Series 8000 – Direct Heat and Water Jacket CO2 incubator (Thermo Scientific, Waltham, MA). When greater than 90% confluency was reached, cells were passaged using 0.25% Trypsin-EDTA (Life Technologies, Carlsbad, CA) to release the

adherent cells from the flask. Depending on the confluency of the cells, cells were passaged at a ratio of 1:10 to 1:20 for general maintenance and continued growth. For transfection, cells were seeded onto a 6-well plate at appropriate densities in 2 ml of media each well 24 hours prior to transfection. When the cells were at around 90% confluency, DNA constructs were transfected into cells using lipofectamine 3000 kit (Invitrogen) and Optimum media (Invitrogen), according to the manufacturer's instructions. At each stage, transfected stable pools were obtained based on antibiotic selection using blasticidin (Invitrogen) for BCHE, Zeocin (Invitrogen) for P14 or P24. Stable pool media was replaced with new media and fresh antibiotics every 3-4 days until cell viability was below 10% in wild-type CHO-K1 cells. Stable pools were then passaged 4-5 times to ensure recovery of cell growth. Stable single clones were established by seeding 0.8 cell/well in 96-well plates (Corning, Tewksbury, MA) for limited dilution.

2.3 IMMUNOBLOTING

Conditioned cell media was harvested from the adherent rhBChE cells, and the supernatant was removed for analysis of secreted protein. For SDS-PAGE, the media samples were stained with SDS loading dye and subsequently denatured by heating at 95oC for 5 minutes. SDS loading dye is composed of 250 mM Tris (pH 6.8), 1% 2-mercaptaethanol (Sigma-Aldrich, St. Louis, MO), 50% glycerol (Sigma-Aldrich, St. Louis, MO), 5% SDS, 0.29% Bromophenol Blue (Sigma-Aldrich, St. Louis, MO), 20 mM Dithiothreitol DDT (Sigma-Aldrich, St. Louis, MO) and stored at -20 ℃ freezer.

Samples were loaded onto 10% polyacrylamide gels, for detection of rhBChE under combinantions of various promoters, his-tag and gene codon optimization. The SDS gels are composed of various amounts of water, 30% Bis-Acrylamide (BIO-RAD, Hercules,

CA), Tris-HCL (Promega, Fitchburg, WI), 10% SDS, 10% APS, TEMED (Invitrogen, Carlsbad, CA). Proteins were separated using gel electrophoresis run at 70V constant voltage through the stacking gel, and then 110V constant voltage through the resolving gel for 90 minutes at room temperature. Proteins were transferred from the gel to nitrocellulose membranes (BIO-RAD, Hercules, CA) by blotting for 75 minutes at constant 100V. Transferred membranes were blocked in 5% milk solution in Phosphate Buffered Saline (Corning Cellgro, Manassas, VA) with 0.1% Tween-20 (PBST) for 1 h. Membranes were washed three with PBST for 10 minutes each time. Membranes were incubated in a 1% milk in PBST solution containing 1:1000 dilution of anti-huBChE rabbit serum (kindly provided by Dr. Ashima Saxena) as primary antibody overnight at 4oC for huBChE detection. Membranes were then washed 4 times with PBST. Membranes were incubated in 1% milk in PBST solutions containing 1:2000 dilution of a horseradish peroxidase-linked anti-rabbit IgG antibody (Amersham, Louisville, CO) as a secondary antibody for 2 hours at room temperature for huBChE detection. After washing the membrane 3 more times, chemiluminescent detection of the bound antibody was achieved using Immun-Star WesternC Chemiluminescent Kit (BIO-RAD, Hercules, CA) on the Molecular Imager® ChemiDoc™ XRS (BIO-RAD, Hercules, CA) with Quantity One Software (BIO-RAD, Hercules, CA).

2.4 NATIVE GEL ELECTROPHORESIS

For native gel electrophoresis, media samples were neither denatured nor reduced. Media samples were stained with a loading dye composed of same chemicals as SDS loading dye described above except for SDS, 2-mercaptaethanol, or 20 mM Dithiothreitol. Buffer conditions for running native gel electrophoresis are run without SDS. HuBChE

activity was detected on the gel by a method developed by Karnowsky and Roots [30]. Briefly, gels were incubated in 100 mM phosphate buffer, pH 7.0, containing 5 mg of thiocholine ester, 0.5 ml of 100 mM sodium citrate, 1.0 ml of 30 mM copper sulphate, and 1.0 ml of 5 mM potassium ferricyanide in a total volume of 10 ml for 20 mins. The gel is then treated with ammonium sulphide solution for 5 mins to form brown bands on the gel.

2.5 Ellman Activity Assay

Purified Conditioned cell media was harvested from adherent CHO-K1 cells. Reagents for Ellman Assay were kindly obtained from Dr. Saxena Ashima. 10 µl of media was added in duplicate to wells of a 96-well microtiter plate (Corning, Manassas, VA), with each well contained 240 µl of 50 mM Phophate Buffer, pH 8.0 and 1.3mM DTNB (PB-DTNB) [31]. Mixture was incubated at room temperature for 20 minutes, and then 50 µl of 6 mM BTC in PB was added. The rate of BTC hydrolysis was read at 412 nm over 10 minutes using a SPECTRAmax PLUS plate reader (Molecular Devices Corp., Sunnyvale, CA) and the SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA). Wells that contained only PB-DTNB without cells were measured as blanks.

2.6 Purification of Butyrylcholinesterase

The rhBChE stabe cell line was seeded at 1x106 million cells per 100mm tissue dish. Let the cells growing for two days until the confluency was above 95%, then change the growth media to Opti-mum reduced serum media (Thermo Fisher Scientific). The cells were incubated for additional two days and then harvested the Opti-mum media and centrifuged to remove cell pellet and collected the supernatant.

For Ni-NTA purification, 200ul 50% Ni-NTA agarose slurry was added into 14ml supernatant and mixed gently by shaking 100 rpm on a rotary shaker for 1 h at room

temperature, then loaded the supernatant-Ni-NTA mixture into a column and collected the flow-through. Wash three times with appropriate volume with washing buffer (20 mM imidazole in PBS solution) and collect wash fractions, and the bound BChE was eluted with 1.5 ml elution buffer (250 mM imidazole in PBS solution). After elution, the eluate was immediately dialyzed with PBS buffer three times using 10 Kd ultrafiltration column (AmiconUltra, Millipore). In the last step of dialysis, collect the sample until the total volume is 300ul. Then the purified rhBChE was evaluated using Ellman assay to determine the active form of rhBChE in different fractions and native electrophoresis followed by K&R staining [30] to determine the isomers of rhBChE after his-tag purification.

2.7 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

After transfecting pcDNA3.1 P24-IRES-GFP into rhBChE stably expressing cell line, stable pools were obtained based on antibiotic selections including blasticidin (Invitrogen) for BCHE, Zeocin (Invitrogen) for P24. And stable pools were passaged 7-10 times to ensure appropriate expression of GFP. Then cells in the stable pool were seeded at 1x106 cells per dish for three dishes. When the confluency was above 90%, cells were washed with PBS (Quality Biological), detached and counted using trypan blue solution 0.4% (w/v) (Invitrogen) to check cell viability. After above 90% confluency, cells were pelleted at 500 g for 15 mins, PBS washed twice and resuspended in 1.5ml PBS in 12 x 75 mm Tube with Cell Strainer Cap (BD Falcon). Cells were carried to JHU medical school to do flow cytometry. After cells with strong GFP expression were sorted, sorted cells were moved to 50 ml tube with culture media up to 40 ml, centrifuge 2100g for 5 mins to remove sorting buffer and culture media and then resuspended cells in a dish with antibiotic selection. Three days later, the sorted cells have more than 50% recovery, which mean a

successful sorting. Passage cells for one more time, then cells were ready for further analysis.

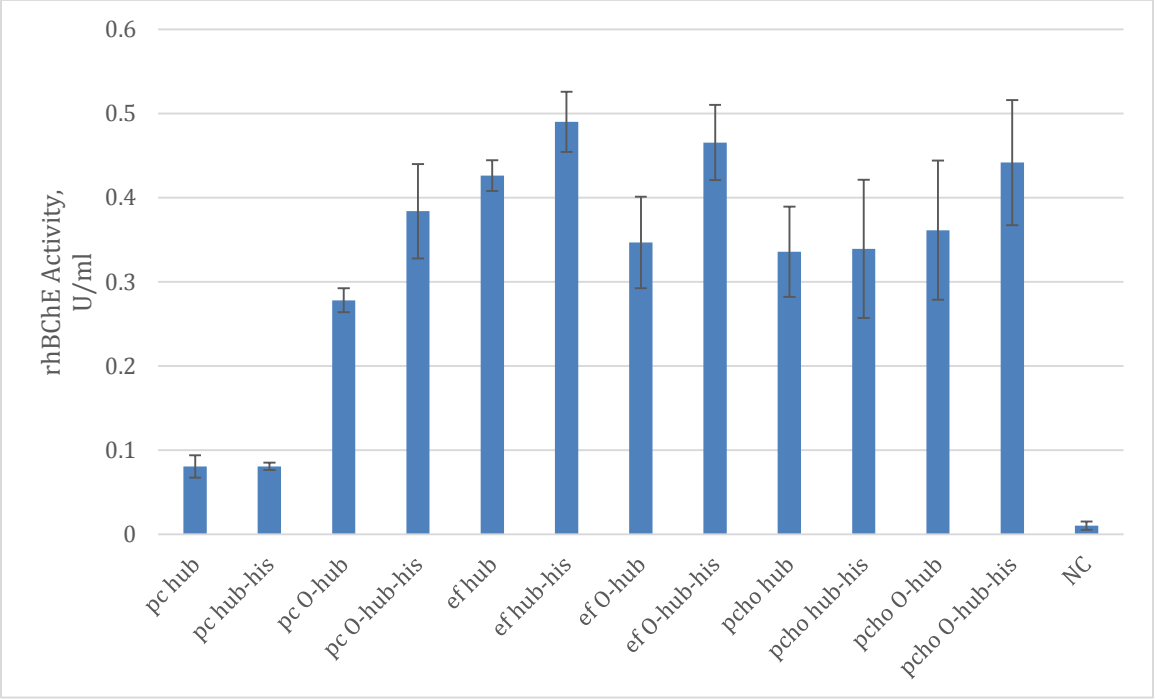
CHAPTER 3: RESULTS

3.1 THE DEVELOPMENT OF AN EFFICIENT RHBCHE EXPRESSION SYSTEM

The first aim of this project is to develop a high-level recombinant human butyrylcholinesterase (rhBChE) expression system. Two human BCHE genes are used, one in which native codons found in human BCHE cDNA and the other with codon optimizations prevalent in CHO genes. Relative performances of these two BCHE genes were evaluated with combinantions of different promoters and his-tag.

Figure 4. Expression of Recombinant HuBChE in CHO.

A



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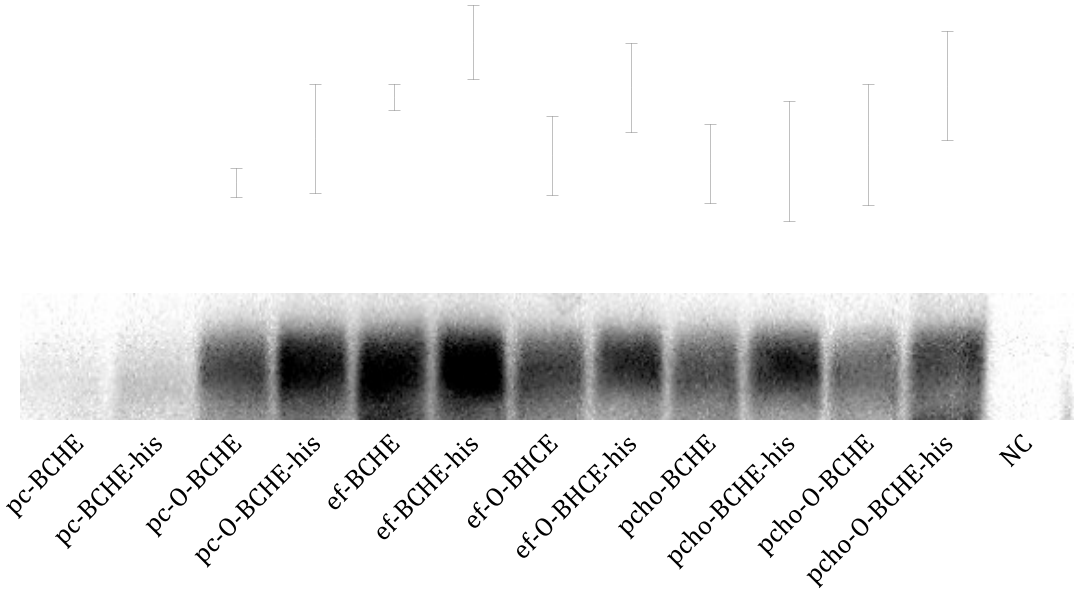

Figure 4. Assessment of BCHE expression levels under various promoters, gene codon optimization and his-tag factors. (A) Ellman Assay detection of huBChE activity; (B) Western Blot detection of HuBChE

Thus, to select an efficient expression system, 12 expression vectors (pcDNA3.1/CMV/BCHE, pcDNA3.1/CMV/BCHE-HIS, pcDNA3.1/CMV/O-BCHE, pcDNA3.1/CMV/O-BCHE-HIS, pEF6/EF/BCHE, pEF6/EF/BCHE-HIS, pEF6/EF/O-BCHE, pEF6/EF/O-BCHE-HIS, pCHO1.0/CMV+EF-1 Hybrid/BCHE, pCHO1.0/CMV+EF-1 Hybrid/BCHE-HIS, pCHO1.0/CMV+EF-1 Hybrid/O-BCHE and pCHO1.0/CMV+EF-1 Hybrid/O-BCHE-HIS) were constructed (Fig. 3) and transiently transfected into CHO-K1 cells via lipofection. Culture media of 12 transfectomas were collected 48 h following the transfection. Conditioned medium of CHO-K1 cell line grown under the same conditions as transfectomas were used as a control. Equal volume of post-

transfection conditioned cell media was loaded into each lane of a 10% polyacrylamide gel to run SDS-PAGE. Following SDS-PAGE, we analyzed the proteins using western blot analysis and the Ellman Activity Assay as shown in Figure 4. A band for recombinant huBChE is observed at the same molecular weight (~80-90 kDa). In addition, there is no band at that molecular weight observed in untransfected wild-type CHO cells, indicating a successful transient transfection of huBChE into CHO. The Western blot analysis was consistent with the Ellman assay results.

According to the Beer-Lambert law, A=εlc, where A is the absorbance measured in optical density (OD/min), ε is the molar adsorptivity, for TNB-, the colorimetric product of DNTB the value is 13260M-1cm-1 [George L Ellman, 1960], l is the pathlengh and the path lengths of the 96-well plate wells containing a total volume of 300 µl (reaction volume) was ~0.32 cm, c is the concentration, mathematical conversion will yield moles of TNB per min and the reaction ratio of BTC:BChE:DNTB is 1:1:1. The activity of purified huBChE was calculated to be ~2100 Units (U)/ml, with one unit of enzyme activity defined as the amount of enzyme that hydrolyzes one micromole of TNB in 1 minute. The results shown in Fig.2A demonstrate that the rhBChE activity of the vector pEF6/EF/BCHE-HIS and pEF6/EF/O-BCHE-HIs were comparable and were equal to approximately 0.49 U/ml and 0.46 U/ml, which is an approximately 6-fold increase compared to the level of human BCHE cDNA with CMV promoter (0.08 U/ml). Interestingly, with CMV promoter, codon optimization can remarkably enhance the expressions of BCHE and BCHE-HIS, which don't have an obvious effect on other promoters. Using codon optimization, the expression levels of BCHE and BCHE-HIS under CMV promoter were 0.28 U/ml and 0.38 U/ml, which showed 3.5-fold and 4.8-fold

increase compared to the levels of BCHE cDNA counterparts respectively. And there was no huBChE activity detected in the WT CHO conditioned cell medium.

Therefore, the vector pEF6/EF/O-BCHE-HIS containing the codon-optimized BCHE gene under the control of the EF promoter (elongation factor 1a) and a C-terminal his-tag was selected and applied in the following experiments as an efficient BCHE expression construct.

3.2 ENHANCEMENT OF RECOMBINANT HUMAN BCHE TETRAMERIZATION IN *VIVO*

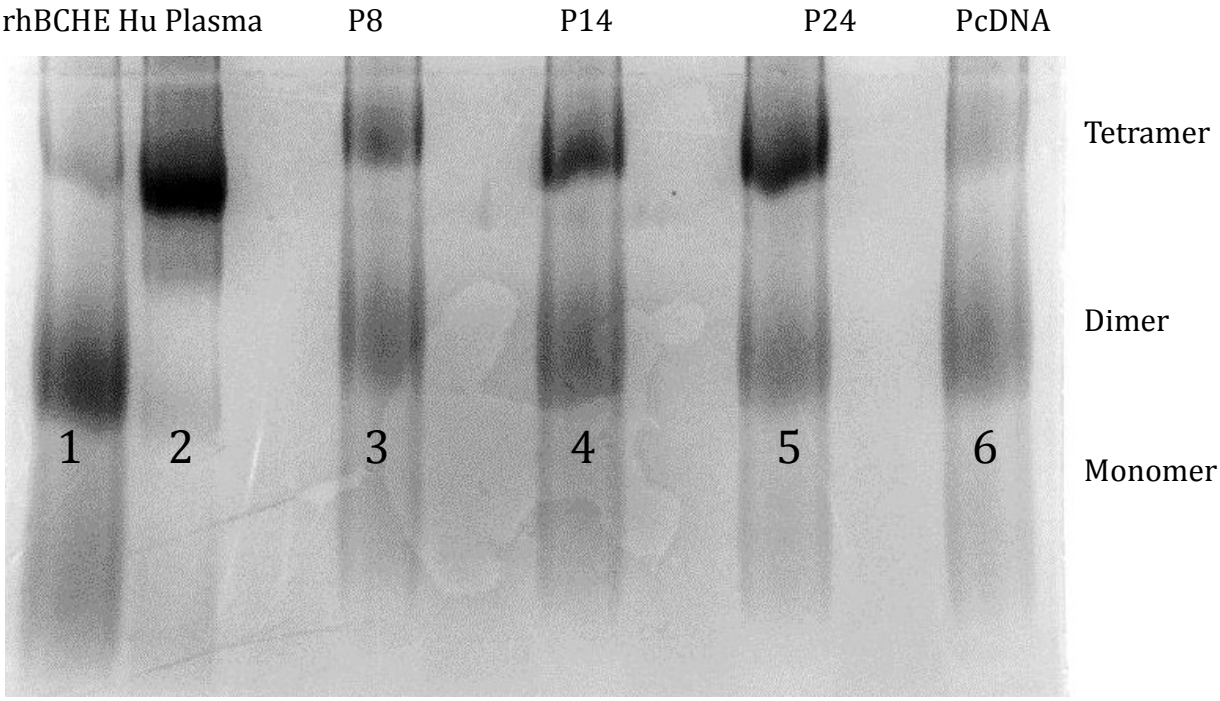

Figure 5 Determine the length of proline-rich sequence required for efficiently assembly of rhBChE into tetramers in CHO cells.

Table 1 Quantification of BChE isomer distribution by determining band intensity using ImageJ analysis.

	P8	P14	P24	NC
Tetramer/%	35.14296	48.36261	59.51934	9.518602
Dimer/%	57.63332	48.51981	35.85448	71.58532
Monomer/%	7.223716	3.117586	4.626178	18.89607

Overexpression of P8, P14 and P24 polyproline sequences in rBChE stably expressing CHO cell line. Two days post-transfection, 2 ml supernatant of each sample was concentrated, dialyzed and then fractionated in a non-denaturing gel. To visualize the migration of rhBChE, the native gels were stained for BChE activity. Lane1, rhBChE from rhBCHE producing CHO cell; Lane2, purified human plasma BChE; Lane3-5, transient expression P8, P14 and P24 sequences on rBChE-prducing CHO cells; Lane6, transient expression of empty PcDNA3.1 vector on rBhCE producing CHO cells.

After tranfection, pEF6/EF/O-BChE-his stable pools were selected under pressure of 10 µg/ml Blasticidin antibiotic, resulting in production of monoclone. Analysis of secreted rhBChE produced by pEF6/EF/O-BChE-his monoclones showed that rhBChE was expressed primarily as dimer (Figure 2, lane1). Thus, the next aim is to enhance rhBChE tetramerization in CHO expression system.

Proline-rich sequences of various poplyproline lengths (P8, P14 and P24) were separately constructed into pcDNA3.1 vector and individually introduced into rhBChE stably expressing cell line. And pcDNA3.1 empty vector was also introduced into the rhBChE stably expressing cell line as a control. As shown in Figure 2, a very remarkable

difference is the increasing band intensity of rhBChE tetramer from P8 to P24 gradually. Furthermore, in order to quantify the content of rhBChE isomers after coexpression of proline-rich sequences, ImageJ analyzer was implemented to quantify the varying band intensity on Fig.2. In Table 1, rhBChE produced in CHO cells were predominantly dimers and a small portion of monomers, which is consistent with some published documents [32]. Approximately 60% of rhBChE was in the tetrameric form after introducing P24 sequence into rhBChE cell line and 48% tetramer and 48% dimer existing after introducing P14 into rhBChE cell line.

3.3 PURIIFICATION OF RECOMBINANT HUMAN BCHE PRODUCED IN CHO CELLS

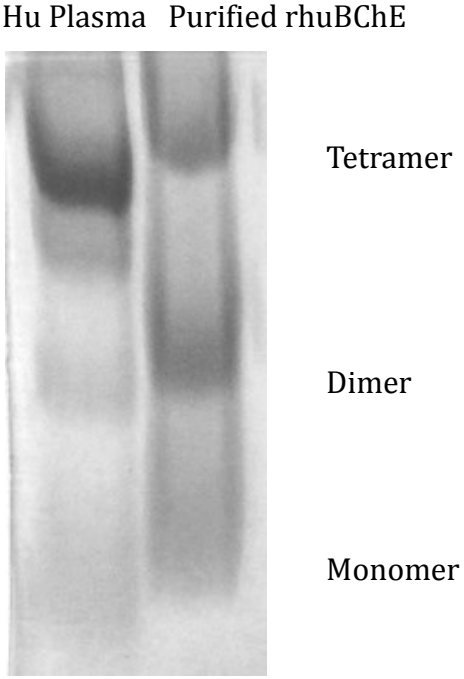

Figure 6 Native Gel Electrophoresis of rhuBChE

Table 2 Ellman Assay result of fractions from Ni-NTA purification

	Load	Flow-through	Washing	Eluate
U/ml	10.49915	1.454327	0.11595	98.9112

Given the Ellman assay results of fractions on the rhBChE purification, the active form of rhBChE was highly enriched in the eluate (98.91 U/ml in 300ul PBS solution) compared to that in the loading supernatant (10.5 U/ml in 14ml supernatant). And there was some rhBChE existing in the flow-through and almost no rhBChE in the washing solution. The recovery of protein purification was above 20%, which is normal for Ni-NTA purification [33].

Moreover, in order to confirm that rhBChE self-assembly is unaffected by Ni-NTA purification process, the purified rhBChE was ran on native electrophoresis and stained by K&R method to distinguish different isomers of purified rhBChE sample. As shown in Fig 3, the isomer distribution of purified rhBChE-his is similar to that of rhBChE supernatant in Fig 2. Thus, Ni-NTA purification with its affinity to his-tagged protein can be used as an effective purification system for purifying rhBChE protein.

3.4 FLUORENCENCE-ACTIVATED CELL SORTING (FACS)

Table 3 Ellman Assay analysis of cell sorted and unsorted rhBChE-P24 stable pool

	sorted bche+p24	unsorted bche+p24	bche	CHOK1
DeltaOD/min	1.0783	0.63705	0.6861	0.00705
U/min	7.623727	4.50403	4.85082	0.049844

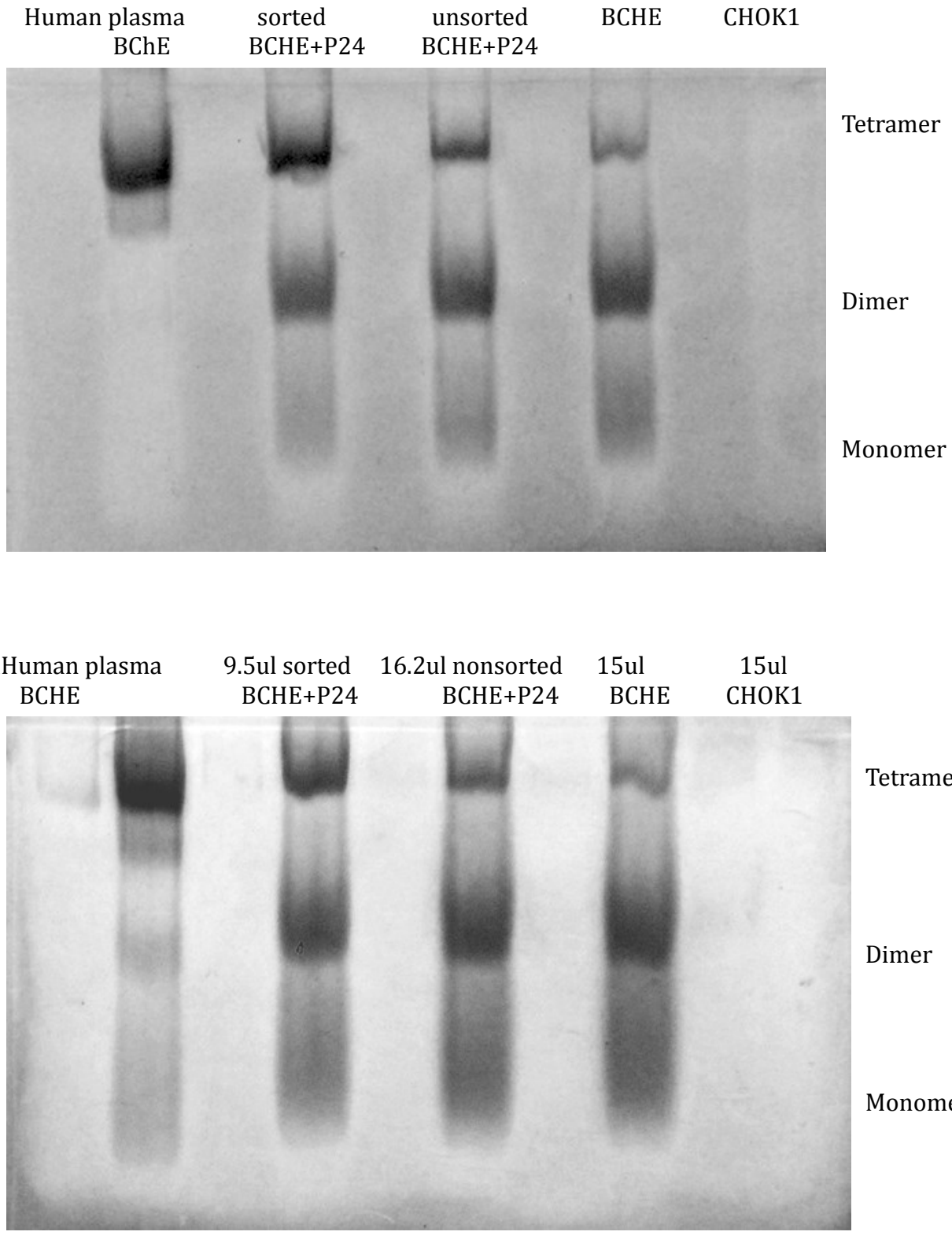

Figure 7 Native electrophoresis of cell sorting effect on rhBChE-P24 stable pool. A: equal loading volume condition B: Equal total activity condition

Next, to further promote rhBChE self-assembly into tetramer, pcDNA3.1 P24-IRES-GFP was constructed and transiently transfected into rhBChE stably expressing cell line. Stable pools were obtained based on antibiotic selections including blasticidin (Invitrogen) for BCHE, Zeocin (Invitrogen) for P24. And stable pools were passaged 7-10 times to ensure appropriate expression of GFP. Then stable pools were sorted using flow cytometry and sorted cells with more than 50% recovery were maintained as rhuBChE-P24 stably expressing cell line.

rhBChE-P24 sorted cell line, rhBChE-P24 unsorted cell line, and rhBChE cell line were seeded at 1x106 cells per dish with 10 ml nutrient media. CHOK1 wild-type cells were also seeded at the same density as a negative control. After the confluency of cells was > 90%, nutrient media was removed and 10 ml Optimum media was added in these four dishes individually. After two days incubation, supernatants (8ml each sample) were collected and centrifuged at 2000 rpm at 4 ℃ until the volume of each sample was 1 ml, then PBS washed three times and the last volume of each sample was 100ul. Meanwhile, Ellman assay was conducted to determine the activity of rhBChE in each supernatant.

After cell sorting, the activity of rhBChE has obviously improved (7.6 U/min) compared to unsorted counterpart (4.5 U/ml). And the activity of unsorted BChE+P24 stable pool (4.5 U/ml), which only performed antibiotic selection, is similar to the activity rhBChE cell line (4.8 U/ml).

Then native electrophoresis was performed based on the same loading volume condition and the same total activity condition respectively. On the equal loading volume basis, in Fig 4 A, sorted and unsorted BChE+P24, BChE and CHOK1 concentrated media were loaded 15 ul individually, and purified BChE from human plasma was loaded 1.5 ul

as positive control. From the result of Fig 4A, sorted BChE-P24 stable pool (Lane 2) has significantly increased the content of BChE tetramer and obviously decreased the contents of dimer and monomer compared to that of unsorted BChE-P24 stable pool (Lane 3). And the introduction of P24 into rhBChE cell line (Lane 3) indeed promoted rhBChE self-assembly into tetramer (Lane 4). Moreover, on the same total activity basis, rhBChE cell line (4.85 U/min, 15 ul loading volume) was chose as a reference. Thus the loading volume of sorted and unsorted BChE-P24 were 9.5 ul and 16.2 ul respectively. And the loading volume of purified human plasma BChE and CHOK1 were kept the same as these in the equal-volume condition above. At the equal total activity condition, the distribution of rhBChE isomers (Fig 7B) showed similar results as that in the equal loading volume condition.

CHAPTER 4: DISCUSSION

Tetramerization of rhBChE not only can enhances enzyme stability and half-life in vivo, but also provides four subunits with each containing an active site that can effectively engage with and inactivate an organophosphorus nerve agent or pesticide [23]. Since endogenously expressed human BChE forms a tetrameric complex with proline-rich peptides and it's not immunogenic [21, 23], we predict that the rhBChE tetramer containing P8, P14 or P24 would behave similarly.

We first studies several factors that may influence the expression of rhBChE in CHO cells, like different promoters, codon optimization effect and his-tag effect. The attempt to use 6Xhistidine tag at the C termini of rhBChE would provide a convenient, economic and robust purification method in BChE purification compared to the commonly used but costly procainamide-affinity purification. The data of Fig 1 A and B demonstrated that his-tag didn't affect the activity or the expression level of rhBChE and the different performance of BChE expression could be attributed to multiple factors such as transcriptional and translational efficiency.

The results of Ni-NTA purification (Figure 4, Table 3) suggested that Ni-NTA purification didn't change the distribution of rhBChE in CHO cells. Collectively, Ni-NTA purification with its affinity to his-tagged protein can be used as an effective purification system for purifying rhBChE protein.

Furthermore, from the result in Fig 2, in the absence of proline-rich sequences coexpression, secreted rhBChE in the culture medium consisted predominantly of the dimeric form (Figure 2, lane 1), and a small amounts of monomeric and tetrameric rhBCHE

were also apparent in the culture medium. These data suggested that the CHO cells do produce a small amount of BChE tetramer, and little is known about whether the existence of rhBChE tetrameric complex is due to the synthesis of a very low abundance of proline-rich peptides in CHO cells or the self-assembly on rhBChE itself.

In addition, comparing that of the untreated control sample (Figure 2, lane 1), the use of P8, P14 and P24 proline-rich sequences increased the abundance of rhBChE tetramers in vivo gradually (Figure 2, lanes 3-5). Whereas P8 was somehow inefficient to provide this function. Proline-rich sequences containing at least 14 proline residues can serve as effective tetramer organizing sequence *in vivo*.

One thing that hamper the formation of rhBChE tetramer was insufficient concentration of P24 secreted in rhBChe-P24 coexpressed cells. Firstly, we tried transfected pcDNA3.1 P24 vector into rhBChE cell line and through 10 times passage with appropriate antibiotic selection and limited dilution, we couldn't obtain a monoclone with high BChE tetramer content. Our hypothesis is that the inefficient tetramer expression of monoclones is attributed to the low expression of P24 and thus antibiotic selection alone is unable to provide a robust rhBChE-P24 expression system. Therefore, we then constructed pcDNA3.1 P24-IRES-GFP vector and try to use fluorescence activated cell sorting method to select high P24 expression cells based on their green fluorescent level.

As shown in Table 3, sorted BChE-P24 stable pool had enhanced BChE activity (7.6 U/min), while unsorted BChE-P24, which were only through antibiotic selections, and rhBChE stable cell line had similar BChE activity, 4.5 U/min and 4.85 U/min respectively. The activity of purified huBChE was calculated to be ~2100 Units (U)/ml, which is consisted of more than 95% tetramer. In Figure 5 A, at the same loading volume condition,

the tetramer content of sorted stable pool was about three-fold higher than that of unsorted stable pool, which means higher P24 concentration assembled more monomers and dimers into tetramers in sorted stable pool. Meanwhile, ompared to BChE cell line, unsorted BChE-P24 stable pool indeed enhanced tetramer assembly to some extent by using antibiotic selection alone, but the secreted P24 content was still insufficient to effectively promote BChE tetrameric formation. Besides, in Figure 5 B, loading at the same total activity, the results again proved that increased concentration of P24 in sorted stable pool significantly promoted tetrameric formation and tetrameric enhancement provided higher enzymatic activity of rhBChE.

CHAPTER 5: CONCLUSION

Recombinant therapeutic proteins have changed the face of modern medicine in the past decade, and they continue to provide innovative and effective therapies for numerous previously refractory illness [34] . Among mammalian systems, the Chinese hamster ovary (CHO) cells are the pre-eminent choice, accounting for nearly 70% of all recombinant protein therapeutics currently produced [34-36]. The CHO cell at its height of technological prominence is related to its ease of transfection and adaptation to growth in various culture conditions, plasticity in the context of genetic alterations, and the capacity to perform post-translational modifications compatible with humans [34, 37-40]. In this context, genetically engineering recombinant human BChE produced in CHO cells has become a good candidate as bioscavenger because it hydrolyzes or scavenges a wide range of toxic compounds including cocaine, organophosphorus pesticides and nerve agents.

This thesis is to develop a recombinant human BChE bioprocess in a laboratory scale. The first aim of this project is to develop an efficient rhBChE expression system in CHO cells. On the exploration of different performance of various promoters, effect of codon optimization and effect of C-terminal his tag together, the vector pEF6/EF/O-BCHE-HIS containing the codon-optimized BCHE gene under the control of the EF promoter (elongation factor 1a) and a C-terminal his-tag was selected as an efficient rhBChE expression construct.

The second aim of this project involves the enhancement of rhBChE self-assembly into tetramers by genetic approach. In the preliminary study, three proline-rich sequences with various proline residues (P8, P14 and P24) were introduced in rhBChE stably expressing cell line and P24 organized approximately 60% of rhBChE into tetramers,

which was chosen as the most efficient rhBChE tetramer organizer compared to other two counterparts. Furthermore, pcDNA3.1 P24-IRES-GFP vector was constructed and fluorescence activated cell sorting was applied to further increase the expression of P24 in rhBChE cell line. After sorting, the P24-rhBChE stable pool was able to stably express rhBChE with a high content of tetrameric isomer and improved enzymatic activity.

We also studied the Ni-NTA purification efficiency to purify rhBChE, which showed 20% recovery rate and did not change the distribution of rhBChE-his isomers in CHO cells. Thus, Ni-NTA purification with its affinity to his-tagged protein can be used as an effective purification system for purifying rhBChE protein.

CHAPTER 6: FUTURE WORK

Two critical factors that determine the BChE enzymatic activity, stability and half-life are tetrameric content and glycosylation. Native BChE in human plasma consists of 95% tetramer [32]. Since we have found an efficient approach to solve the low-tetramer issue in recombinant human BChE, the next step would be to optimize the glycosylation pattern of rhBChE in CHO cells. I have outlined the main plans for the future study as below.

6.1 FURTHER ENHANCE RHUBCHE TETRAMERIZATION BY EUKARYOTIC TRANSLATION INITIATION FACTOR 5A-1

Eukaryotic initiation factor 5A (eIF5A) is a small, acidic protein essential for cell proliferation, which represents the only known eukaryotic protein activated by posttranslational hypusination [41]. eIF5A is highly conserved throughout eukaryotes. Sequence conservation is especially high around the hypusine residue, underlining the meaning of this unusual protein modification throughout eukaryotic evolution. Although eIF5A is intimately involved in eukaryotic cell proliferation, the true physiological function of this essential factor has yet to be elucidated. Park et al. [42]mentioned that eIF5A may be a bimodular protein interacting with both RNA and proteins, and is presumed to have an important role in the translation machinery.

Thus the next step we will transiently transfect elf5A gene in rhuBChE-P24 sorted cells and examine the content of rhuBChE tetramerization using native electrophoresis.

6.2 TEST THE STABILITY OF PURIFIED RHUBCHE TETRAMERS CHAPERONED WITH P24

Protein stability is important for its storage and transportation. And the repeated freeze-thaw cycles will decrease protein stability. Thus we want to test the stability of purified rhuBChE tetramer chaperoned with P24 using repeated slow freeze and fast thaw cycles [43]. Also with the assistance of native electrophroresis, we will determine the degradation of rhuBChE tetramer due to the numbers of freeze and thaw cycles.

6.3 GLYCAN ANALYSIS

After protein purification, the *N*-linked oligosaccharides will be cleaved from rhuBChE and treated with various enzymes to determine the Sialic Acid/Galactose ratio. Then we will examine the alpha (2-3)-sialic acid content on rhuBChE glycans of the engineered cell lines. We will treat purified recombinant proteins with an alpha (2-3)-specific neuraminidase to remove alpha (2-3)-linked sialic acids. The resulting glycans will be removed from the peptides using glycosidases and analyzed using multi-dimensional mass spectrometry (MS).

In the future, we hope to be able to perform *in vitro* activity and OP inhibition assays with our recombinant huBChE to see the effects of the genetically-engineered CHO cell lines. Ultimately, we want to examine the *in vivo* circulatory lifetime of rhuBChE, beginning with animal trials.

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- Engineered Chinese Hamster Ovary cells to produce butyrylcholinesterase, with corrected glycan structures.
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SKILLS

- **Cell Culture** in suspension and adherent cell lines (CHO-S, CHO-K1, HEK 293), Cell Cryopreservation
- **Cell Line Engineering**: Transient and Stable DNA Transfection, Gene knockdown using siRNA Transfection, Clone Selection
- **Molecular Cloning**: Vector design and construction, PCR, RT-PCR, colony PCR, Gene and Primer Design, DNA/RNA extraction and purification, DNA Gel Electrophoresis, Transformation
- **Protein Purification and Characterization**: SDS-PAGE, Western Blot Analysis, Lectin Blot Analysis, Immunoprecipitation, Ni^{2+} Magne-His Purification, Ultracentrifugation, Tangential Flow Filtration (Diafiltration), UV/Vis Spectroscopy, ELISA, Enzymatic Assays, BCA Assay

- **Proteomics and Glycoproteomics**: Solid-phase extraction of N-linked glycopeptides, Protein digestion for Mass Spectrometry Analysis
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PUBLICATIONS

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